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Bone morphogenetic protein-7 release from endogenous neural precursor cells suppresses the tumourigenicity of stem-like glioblastoma cells

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Glioblastoma cells with stem-like properties control brain tumour growth and recurrence. Here, we show that endogenous neural precursor cells perform an anti-tumour response by specifically targeting stem-like brain tumour cells. *In vitro*, neural precursor cells predominantly express bone morphogenetic protein-7; bone morphogenetic protein-7 is constitutively released from neurospheres and induces canonical bone morphogenetic protein signalling in stem-like glioblastoma cells. Exposure of human and murine stem-like brain tumour cells to neurosphere-derived bone morphogenetic protein-7 induces tumour stem cell differentiation, attenuates stem-like marker expression and reduces self-renewal and the ability for tumour initiation. Neurosphere-derived or recombinant bone morphogenetic protein-7 reduces glioblastoma expansion from stem-like cells by down-regulating the transcription factor Olig2. *In vivo*, large numbers of bone morphogenetic protein-7-expressing neural precursors encircle brain tumours in young mice, induce canonical bone morphogenetic protein signalling in stem-like glioblastoma cells and can thereby attenuate tumour formation. This anti-tumour response is strongly reduced in older mice. Our results indicate that endogenous neural precursor cells protect the young brain from glioblastoma by releasing bone morphogenetic protein-7, which acts as a paracrine tumour suppressor that represses proliferation, self-renewal and tumour-initiation of stem-like glioblastoma cells.

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Abbreviations: BMP = bone morphogenetic protein; CD133 = prominin-1; GFP = green fluorescent protein; GSC = glioma stem cell; PCR = polymerase chain reaction

Introduction

Neural stem and precursor cells of the adult brain contribute to plasticity in the hippocampus and may provide a cellular source for brain repair (Zhao *et al*., 2008). However, the stem cell niches may also be the point of origin for highly aggressive brain tumours, namely glioblastoma (Holland, 2001; Sanai *et al*., 2005). A subpopulation of glioblastoma cells with stem-like properties probably controls the course of pathology and may also account for tumour-relapse (Galli *et al*., 2004; Kondo *et al*., 2004; Singh *et al*., 2004). These stem-like brain tumour initiating cells [previously defined by others as glioma stem cells (GSC); Clarke *et al*., 2006] are characterized by their potential for self-renewal, maintenance of a cellular hierarchy and increased tumourigenicity, as compared to bulk tumour cells (Stiles and Rowitch, 2008; Jordan, 2009). GSCs can be identified in primary patient material and from various glioblastoma cell cultures by a range of different markers (Singh *et al*., 2004; Bleau *et al*., 2009; Son *et al*., 2009), of which prominin-1 (CD133) is most commonly used (Stiles and Rowitch, 2008). We and others have previously established that endogenous neural precursor cells from the subventricular zone and white matter migrate towards experimental glioblastoma (Glass *et al*., 2005; Assanah *et al*., 2006, 2009; Walzlein *et al*., 2008). Here we show that bone morphogenetic proteins (BMPs), which control neurogenesis and gliogenesis in the subventricular zone (Lim *et al*., 2000; Colak *et al*., 2008), co-function as paracrine tumour suppressors in the CNS. Neural precursor cells release BMP7, which down-regulates Olig2 in stem-like glioblastoma cells and attenuates the tumourigenicity of GSCs. This anti-tumour response is efficient in young brains but declines with ageing.

Materials and methods

Animals

All animals were handled according to governmental and internal (Max Delbrück Center) rules and regulations. Wild-type C57BL/6 and non-obese diabetic-severe combined immunodeficiency mice (Charles River Breeding Laboratories; Schöneiche, Germany) were housed with a 12 h light/dark cycle and received food *ad libitum*. Nestin-green fluorescent protein (GFP), BMP7-lacZ animals and surgical procedures were described previously (Godin *et al*., 1998; Glass *et al*., 2005; Walzlein *et al*., 2008).

Cell culture

The human glioblastoma cells GBM1 (previously designated NCH421K), GBM2 (also designated B4), GBM3 (previously designated NCH441) and GBM4 (previously designated NCH644) were derived from tumour resections. Mouse astrocytoma cells were obtained from the National Cancer Institute, NCI-Frederick (MD, USA). All brain tumour cells were maintained as described for neurospheres below.

Mouse neurospheres were gained from the microdissected and dissociated subventricular zone, and were propagated in neurobasal medium containing supplement (B27), growth factors (20 ng/ml epidermal and fibroblast growth factors from PeproTech, Hamburg, Germany) and additives (0.2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin; from Sigma, St. Louis, MO, USA). Microglia (Hoffmann *et al*., 2003), astrocytes (Pivneva *et al*., 2008) and neurons (Chirasani *et al*., 2009) were all cultivated as described.

Lentiviral vectors

Retroviral vectors for the expression of GFP, Olig2-IRES-GFP or Olig2VP16-IRES-GFP have been described previously by others (Hack *et al*., 2005). These vectors were pseudotyped with the vesicular stomatitis virus-G envelope to generate lentiviral expression vectors.

Flow cytometry

Fluorescence-activated cell sorting analysis was carried out on a LSR II (BD Biosciences, Erembodegem, Belgium). Cell sorting was carried out using a fluorescence-activated cell sorting-Aria-II (BD Biosciences). Antibodies for murine CD133, human CD133 and human isotype control were all from Miltenyi Biotech (Bergisch Gladbach, Germany). Mouse isotype control was from R&D Systems (Heidelberg, Germany). Antibodies for BMP-receptor Ib, BMP-receptor II and isotype controls were from RnD Systems, (Wiesbaden, Germany). Data were analysed using CellQuest and FACSDiva (BD Biosciences) and FlowJo software (Treestar, Ashland, OR, USA).

Enzyme-linked immunosorbent assay

The Quantikine BMP7 enzyme-linked immunosorbent assay kit was purchased from R&D-Systems, used according to the manufacturer's instructions and analysed using an Infinite-M200 plate reader (Tecan, Mainz, Germany).

Cytotoxicity assay

Cell death was studied using the Cytotox-Fluor kit from Promega (Mannheim, Germany) according to the manufacturer's instructions.

Immunohistochemistry

The paraformadehyde perfused cryoprotected brains were rapidly frozen in dry ice and mounted onto a sliding microtome. 40 μm thick sections were incubated for 48 h at 4°C with relevant primary antibodies (Kempermann *et al*., 2003; Glass *et al*., 2005). Antibody against p-Smad1/5/8 was from Abcam (Cambridgeshire, UK), antibodies for vimentin (clone V9), BMP2/4 and BMP7 were from Santa Cruz Biotechnology, Heidelberg, Germany. All other antibodies have been described previously (Glass *et al*., 2005; Walzlein *et al*., 2008).

Microscopy

For confocal microscopy (LSM5 EXITER, from Zeiss, Jena, Germany) we used appropriate gain and black level settings (determined on control tissues stained with secondary antibodies alone). All confocal images were taken with a 40× magnification objective. Fluorescence microscopy for rhodamine-red labelled vimentin and diaminobenzidine labelled p-Smad1/5/8 were recorded as single images with a Zeiss Axioplan microscope, and images were overlaid using Photoshop-CS software (Adobe, San Jose, CA, USA).

Reverse transcriptase polymerase chain reaction

RNA was isolated by using the RNAeasy-kit from Qiagen (Hilden, Germany); all buffers and enzymes were purchased from Invitrogen (Karlsruhe, Germany) and oligonucleotides were from MWG-Biotech (Ebersberg, Germany). First-strand complementary DNA was synthesized with Moloney murine leukaemia virus reverse transcriptase using 1 μg RNA and oligo dT primer; gene-specific primers were used as described by others (Piccirillo *et al*., 2006).

Real-time polymerase chain reaction

Total RNA of cultured cells was isolated using Trizol (Invitrogen) and 5 μg total RNA was reverse transcribed using Superscript II (Promega), according to the instructions by the manufacturers. Quantitative real-time polymerase chain reaction (PCR) was performed on the iCycler IQ 5 multicolour real-time detection system (Bio-Rad, Munich, Germany), using absolute SYBR green fluorescein (ABgene, Hamburg, Germany). Oligonucleotides were purchased from Invitrogen.

A normalized complementary DNA panel of pathologist-verified human brain tumours (OriGene Technologies, Inc., Rockville, MD, USA) was used to probe for Olig2 and Bmp7 transcript levels by quantitative real time PCR.

Western blot

Cells were lysed in HEPES-sodium-Triton-glycerol-buffer [20 mM HEPES pH 7.5, containing 150 mM NaCl, 1% (w/v) Triton X-100 and 10% (w/v) glycerol]; the protein lysate was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were incubated with specific antibodies and western blots were developed using the chemiluminescence method (GE-Healthcare, Freiburg, Germany). Antibody for (non-phosphorylated) Smad1/5/8 was from Abcam; antibodies for p-Smad1/5/8, p-Smad2 and anti-Smad 2/3 were from Cell Signalling, antibodies for α-tubulin and actin were from Sigma, Munich, Germany.

Statistical analysis

Survival statistics were analysed using MATLAB software (Natick, MA, USA). Bar diagrams are shown as mean values ± standard errors of the mean (SEM). Comparisons among the groups were performed with the Student's *t*-test.

Results

BMP7 is constitutively released from neural precursor cells

Endogenous and exogenous neural precursor cells migrate to experimental brain tumours *in vivo* and fulfill some—as yet undefined—tumour suppressor function (Staflin *et al*., 2004; Glass *et al*., 2005; Assanah *et al*., 2006; Walzlein *et al*., 2008). Tumourigenicity of primary brain tumours like glioblastomas is controlled by GSCs (Jordan *et al*., 2006; Jordan, 2009) and the present study indicates that neural precursor cells can specifically suppress these stem-like glioblastoma cells. In order to investigate the molecular basis of the anti-tumour effect of neural precursor cells against GSCs in detail, we used GSC cultures gained from different primary glioblastoma multiforme resections; information on glioblastoma patients from which samples were retrieved is given in Supplementary Fig. 1. These GSCs were maintained under serum-free conditions in the presence of epidermal and fibroblast growth factors, formed multi-cellular spheres, were clonogenic, expressed an established set of stem- and progenitor-cell markers and reverted to an adherent and neuronal/glial-differentiated phenotype under differentiating conditions (Supplementary Fig. 1 and Supplementary Table 1). Cultivated GSCs had genomic features typical for glioblastoma multiforme and retained the chromosomal aberrations of the parental tumour (Ernst *et al*., 2009). GSCs could be highly enriched from glioblastoma multiforme cultures (93–99% purity), as evidenced by using CD133 as a GSC marker (Supplementary Fig. 2). Undifferentiated CD133(+) GSCs had very high tumour initiating capacity in mice *in vivo* as compared to their differentiated, CD133(−) counterparts. In addition to the primary human glioblastoma multiforme cultures (which were investigated in immunodeficient mouse models), we have established purification of CD133(+) GSCs from a mouse astrocytoma line (GL261 cells) that is syngenic with our immunocompetent mouse models (Supplementary Figs. 1 and 2, Supplementary Table 1). Altogether, we established that CD133 is a valid GSC marker in all our tumour cell cultures, since the CD133(+) but not the CD133(−) brain tumour cells had capacity for self-renewal, maintained a cellular hierarchy and had increased tumourigenic potential (Stiles and Rowitch, 2008).

Various BMPs were previously detected in the neural stem cell niche (Lim *et al*., 2000; Colak *et al*., 2008) and recombinant BMP2 or BMP4 may be of clinical use against GSCs (Piccirillo *et al*., 2006; Lee *et al*., 2008). We screened BMP-expression in neural precursor cells in order to identify BMPs that may function as GSC-specific tumour-suppressors in the CNS. We compared the expression levels of all brain related BMPs: BMP1, BMP2, BMP3, BMP4, BMP5, BMP6, BMP7 and BMP8b (Mehler *et al*., 1997; Ebendal *et al*., 1998) in murine astrocytoma, in murine GSCs (see Supplementary material and Wu *et al*., 2008), in murine neurospheres and in differentiating neurospheres (cells undergoing differentiation into astrocytes, oligodendrocytes and neurons) by using quantitative real-time PCR. We found that BMP7 is the predominant BMP in neurospheres and that BMP7 is more

strongly expressed in neurospheres than in any other sample investigated (Fig. 1A).

Our enzyme-linked immunosorbent assay showed that BMP7-protein is abundant in neurospheres, but not in astrocytes, microglia or neurons. Migratory polysialic acid-neural cell adhesion molecule-positive neural precursor cells in particular contain BMP7 (Fig. 1B). We observed that the BMP mRNA expression profile was in agreement with BMP-protein release from neural precursor cells and found that BMP7, but not BMP2 or BMP4, was constitutively released at a detectable level from neural precursor cells (Fig. 1C). Altogether our data show that the migratory fraction of neural precursor cells pre-dominantly expresses and constitutively releases BMP7.

Neural precursor cell-released BMP7 induces canonical BMP signalling and differentiation in stem-like glioblastoma cells

Primary human glioblastoma cells, which were highly enriched in CD133(+) GSCs (GBM1, previously designated NCH421K, see Supplementary Fig. 1), express the type-I and type-II BMP-receptors (BMP-receptor I, with the Ia and Ib subtype and BMP-receptor II; Fig. 2A), which are both necessary for BMP signalling (Massague, 2000; Lee *et al*., 2008). Likewise, FACS-analysis revealed that CD133 positive GSCs express BMP-receptor Ib and BMP-receptor II on the plasma-membrane (Supplementary Fig. 3A). Western blotting showed that neural precursor cell-conditioned medium and recombinant BMP7 activate BMP signalling in GSCs (Fig. 2B; phosphorylation of Smad1/5/8 protein; p-Smad1/5/8; Massague, 2000). Smad-phosphorylation is rapidly, within 15 min and peaking at 30 min, induced in GSCs (Supplementary Fig. 3B). The selective BMPR-I antagonist compound C (also termed dorsomorphin; Yu *et al*., 2008) completely abrogated the effect of neural precursor cell-conditioned medium and BMP7 on Smad1/5/8 phosphorylation. Neural precursor cells induced BMP signalling in GSCs specifically via release of BMP7 since the effect of neural precursor cell-conditioned medium on p-Smad1/5/8 could be blocked by a BMP7-specific blocking antibody, but not with antibodies inhibiting BMP2 or BMP4 (Fig. 2C). Furthermore, we observed that neural precursor cell-stimulated p-Smad1/5/8 in GSCs initiates transcription of well described Smad1/5/8 target genes like DNA-binding protein

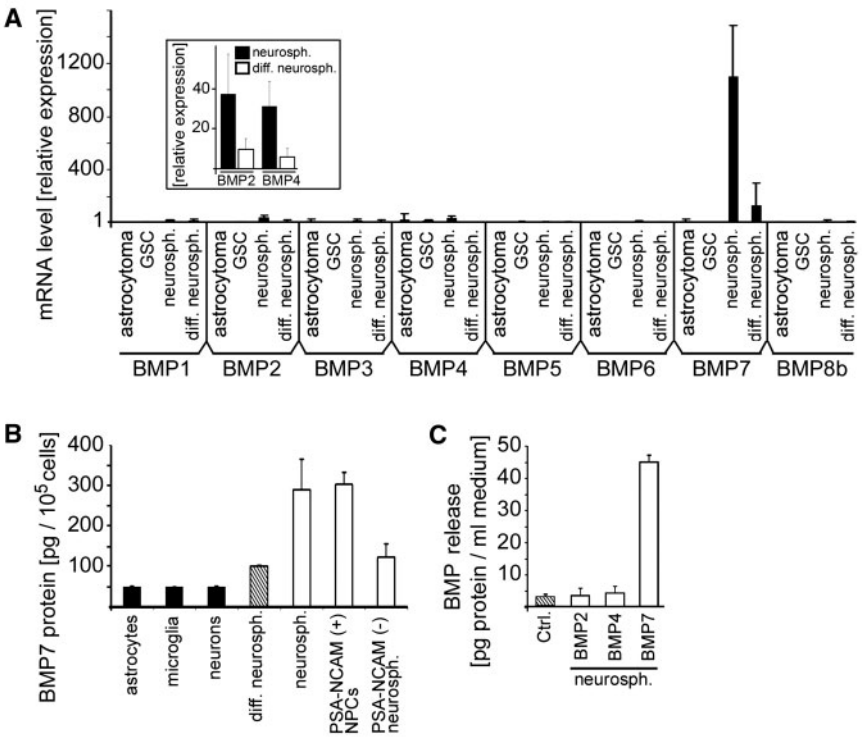

Figure 1 BMP7 is predominantly expressed in polysialic acid-neural cell adhesion molecule-positive neural precursor cells *in vitro* and constitutively released from neurospheres. (**A**) Mouse astrocytoma cells (GL261 cells), CD133-expressing mouse astrocytoma cells (GSCs) and neurospheres were all cultured under neurosphere conditions. Additionally, neurospheres were induced to differentiate (diff-neurosph.); all cell culture samples were analysed for the expression of the indicated BMPs by real-time PCR; BMP-expression levels in the cell type with the lowest expression were arbitrarily set as one. Insert in (**A**): expression of BMP2 and BMP4 is shown by using a different scale. (**B**) BMP7 expression was investigated by enzyme-linked immunosorbent assays in mature astrocytes neurons, microglia, differentiated neural precursor cells (NPCs) and neurospheres. Additionally, polysialic acid-neural cell adhesion molecule (PSA-NCAM) (+) and (−) cells were fluorescence-activated cell sorting-purified and BMP7 expression was compared between both samples. (**C**) Neurospheres were cultured for 3 days and cell culture supernatants (conditioned medium) was assayed (by enzyme-linked immunosorbent assay) for BMP2, BMP4 and BMP7 (as compared with cell-free medium).

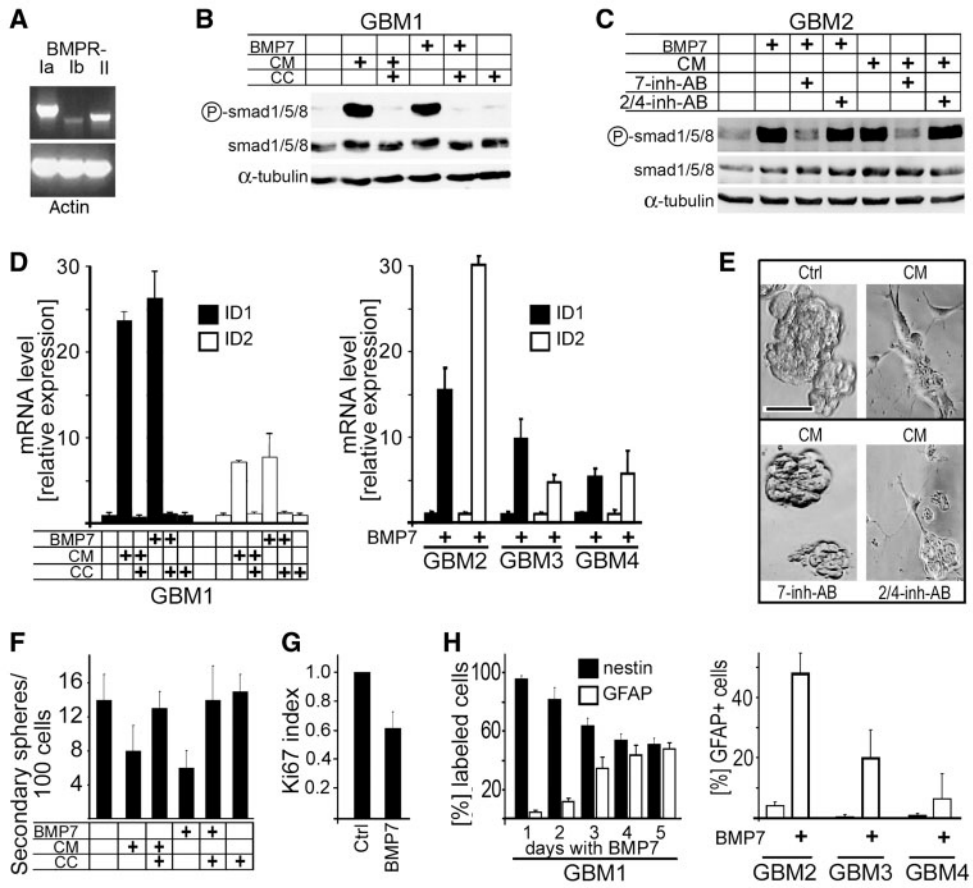

Figure 2 Neurosphere derived BMPs induce differentiation and attenuate self-renewal of GSCs. Human glioblastomas (GBM1, GBM2, GBM3 and GBM4) were cultivated, CD133(+) cells were enriched (to minimum 93% purity) and these GSCs were investigated for neural precursor cell-induced BMP signalling (**A–D**), self-renewal and differentiation (**E–H**). (**A**) Reverse-transcriptase-PCR shows that GBM1 GSCs express BMP-receptors (BMPR) type-Ia, -Ib and -II. (**B**) GBM1 GSCs were stimulated with recombinant BMP7 or neural precursor cell-conditioned medium (CM), with or without the BMP-receptor antagonist compound C (CC); BMP-responses (Smad1/5/8-phosphorylation) were investigated by western blotting. (**C**) GBM2 GSCs were stimulated with BMP7 or conditioned medium, with or without an inhibitory antibody specifically blocking BMP7 (7-inh-AB) or inhibitory antibody with a dual specificity for BMP2 and BMP4 (2/4-inh-AB); Smad1/5/8-phosphorylation is shown. (**D**) GBM1 GSCs were stimulated as described in **B** and analysed by real-time PCR for expression of the Smad targets DNA-binding protein inhibitor (ID)2; GSCs from GBM2, GBM3 and GBM4 were stimulated with BMP7 (or left untreated) and analysed for DNA-binding protein inhibitor1 and 2. (**E**) GBM2 cells undergo morphological changes (i.e. tumour spheres become adherent) after incubation with conditioned medium; BMP7-, but not BMP2/4-inhibitory antibody, blocks morphological changes. (**F**) GBM1 GSCs were treated as in **B** and formation of secondary spheres was quantified. (**G**) GBM1 GSCs were stimulated with BMP7 (or left untreated) and proliferation was quantified. (**H**) GBM1 GSCs. were stimulated with BMP7 and markers of undifferentiated (nestin) and differentiated (glial fibrillary acidic protein; GFAP) cells were monitored over a time-course; likewise GBM2, GBM3 and GBM4 were investigated for BMP7 mediated effects on glial fibrillary acidic protein expression. Scale bar in **E**: 60 μm.

inhibitor (ID) 1 and ID2 (Massague, 2000), as evidenced by quantitative PCR (Fig. 2D). BMP7 induced ID1 and ID2 expression in all GBMs studied (GBM3 cells were previously designated NCH441 and GBM4 were named NCH644, see also Supplementary Fig. 1).

Our subsequent experiments indicated that neural precursor cell-released BMP7 induced differentiation of GSCs. It was readily observable that GBM-spheres became adherent after stimulation with neural precursor cell-conditioned medium, which could be prevented by incubation with an inhibitory antibody for BMP7, but not for BMP2/4 (Fig. 2E). Furthermore, neurosphere-conditioned medium and recombinant BMP7 interfered with stem cell-properties in other *in vitro* assays (investigating the unlimited capacity for self-renewal and maintenance of a cellular hierarchy; Jordan *et al*., 2006; Jordan, 2009). First, we noted that transient stimulation with neural precursor cell-supernatant and recombinant BMP7 had a persistent effect on GSC expansion in a sphere formation assay: we stimulated GSCs with BMP7 or neural precursor cell-conditioned medium (± compound C, or compound C alone), plated the cells at low density (100 cells per group) to exclude paracrine signalling and determined the number of secondary spheres: recombinant BMP7 and neural precursor cell-conditioned medium attenuated secondary sphere formation of GSCs (Fig. 2F). Moreover, addition of

BMP7 to GSCs reduced proliferation by 40% (Fig. 2G). The number of undifferentiated, nestin-expressing cells was reduced by 50% after treating GBM1 cells with BMP7 over a 5 day time-course (Fig. 2H). Concurrently, we counted a 45% increase in more differentiated, GFAP expressing cells. Differentiating effects of BMP7 on GSCs were also seen in all other glioblastoma multiforme cells studied.

Altogether, this indicates that BMP7 release from neural precursor cells induced canonical BMP signalling and thereby caused GSC differentiation.

Neural precursor cell-derived BMP7 attenuates expression of a glioblastoma stem cell marker and suppresses GSC function

Expression of CD133 on GSCs correlates with tumour initiating ability (Singh *et al*., 2004) and the mean fluorescence intensity for CD133 (by fluorescence-activated cell sorting) gives a first estimate for the abundance of GSC in tumour samples (Piccirillo *et al*., 2006; Lee *et al*., 2008). We exposed both human and mouse GSCs to neural precursor cell-derived BMP or recombinant BMP7 and quantified the number of stem-like brain tumour cells (Fig. 3A–D). Recombinant BMP7 reduced the CD133 fluorescence level to 45.2, 44.1 and 66.3% of control levels in human-GBM1, human-GBM2 and mouse–mouse astrocytoma cells, respectively (Fig. 3A, B and D). The conditioned medium from neurospheres had an even more profound effect on the CD133 fluorescence level (reduction to 33.3, 31.3 and 61.4%, respectively, of control levels in GBM1, GBM2 cells or mouse astrocytoma cells). The effect of both the recombinant BMP7 and neurosphere conditioned medium was always efficiently antagonized by compound C and was independent of glioblastoma-cell plating densities. Compound C alone did not significantly influence CD133 expression levels. Importantly, we found that the effect of neural precursor cell conditioned medium on CD133(+) GSCs was mediated specifically by BMP7 (Fig. 3B). The natural BMP-antagonist noggin (Massague, 2000) also highly effectively blocked the neurosphere-conditioned medium induced down-regulation of CD133 in murine GSC (Fig. 3D). To investigate if the loss of CD133(+) cells from glioblastoma cultures also reflects reduced tumourigenicity by GSCs, we performed a survival study. Therefore, CD133(+) mouse astrocytoma cells were either treated for five days with recombinant BMP7 or cultivated in control-medium before inoculation into mice. In comparison to classical tumour cell models, we only injected 100 cells per animal—this procedure indicates that the inoculum contained brain tumour cells with a high potential for tumour-initiation (Singh *et al*., 2004; Wu *et al*., 2008). As shown in Fig. 3E mice survived significantly longer when inoculated with BMP7 treated cells as compared to controls.

Altogether our data show that the neural precursor cell-derived BMP7 can strongly attenuate GSC-marker expression and GSC function.

Neural precursor cell-derived BMP attenuates stem-like glioblastoma cells expansion via down-regulation of Olig2

Our series of experiments indicated that GSCs lose their stem-like properties after exposure to neural precursor cell-derived BMP7. The transcription factor Olig2 is expressed in the vast majority of proliferating high-grade brain tumour cells, Olig2 mRNA-expression levels are increased in glioblastomas (as compared to lower grade tumours; Supplementary Fig. 5); it is ubiquitous in stem-like glioblastoma cells and mandatory for glioblastoma-initiation (Ligon *et al*., 2007).

Here, we demonstrate that stimulation of GSCs with neural precursor cell-derived BMP or with recombinant BMP7 down-regulated Olig2 expression levels, as determined by quantitative PCRs (Fig. 4A); BMP7 efficiently down-regulated Olig2 in all GBMs studied. To investigate if Olig2 has a direct impact on the stem-like identity of GSC we used lentiviral gene transfer to over-express Olig2 or a dominant-negative form of Olig2 (Olig2-VP16) in GBM1 stem-like glioblastoma cells (Fig. 4B). First, we investigated if Olig2 expression relates to GSC expansion, which would be in line with its previously suggested function in facilitating progression through the cell-cycle (putatively by blocking $p21^{waf1}$ function; Stiles and Rowitch, 2008). Therefore, we transduced GBM1 cells with vectors for Olig2 or Olig2-VP16 and plated them at low density. These cells were then maintained in medium with or without BMP7 for five days, transferred into fresh (BMP7-free and non-conditioned) stem cell medium and subsequently secondary sphere formation was monitored. We observed that addition of BMP7 reduced secondary sphere formation in GBM1 cells expressing GFP (as expected from our previous experiments), but that Olig2 over-expression alone increased the number of spheres by 66% (as compared to controls) and blunted the effect of BMP7 (Fig. 4C). Furthermore, blockade of Olig2 function (by Olig2-VP16) reduced secondary sphere formation *per se* and application of BMP7 did not have any additive effect. Altogether, these data suggest that Olig2 is a downstream effector of BMP7 signalling. However, Olig2 does not directly control CD133 expression and the stem-like identity of GSC, but has profound effects on the ability of GSCs to expand from single cells into entire spheres (Fig. 4D). In agreement with this interpretation we observed that the first cell division of BMP7-treated GSCs and Olig2-VP16 over-expressing GSCs was often abortive and either directly generated stalled or dead cells, or produced one apparently normal cell connected with a non-viable cell (not shown). Hence, our data indicate that the BMP7-mediated reduction in GSC proliferation and the induction of GSC cell death can be caused by reduced expression of Olig2.

Neural precursor cells accumulate at glioblastoma *in vivo* and express BMP7

Since neural precursor cells can suppress GSCs *in vitro* and since brain-endogenous neural precursor cells interact with experimental brain tumours *in vivo* (Glass *et al*., 2005; Walzlein *et al*., 2008), we asked whether neural precursor cells may have an intrinsic

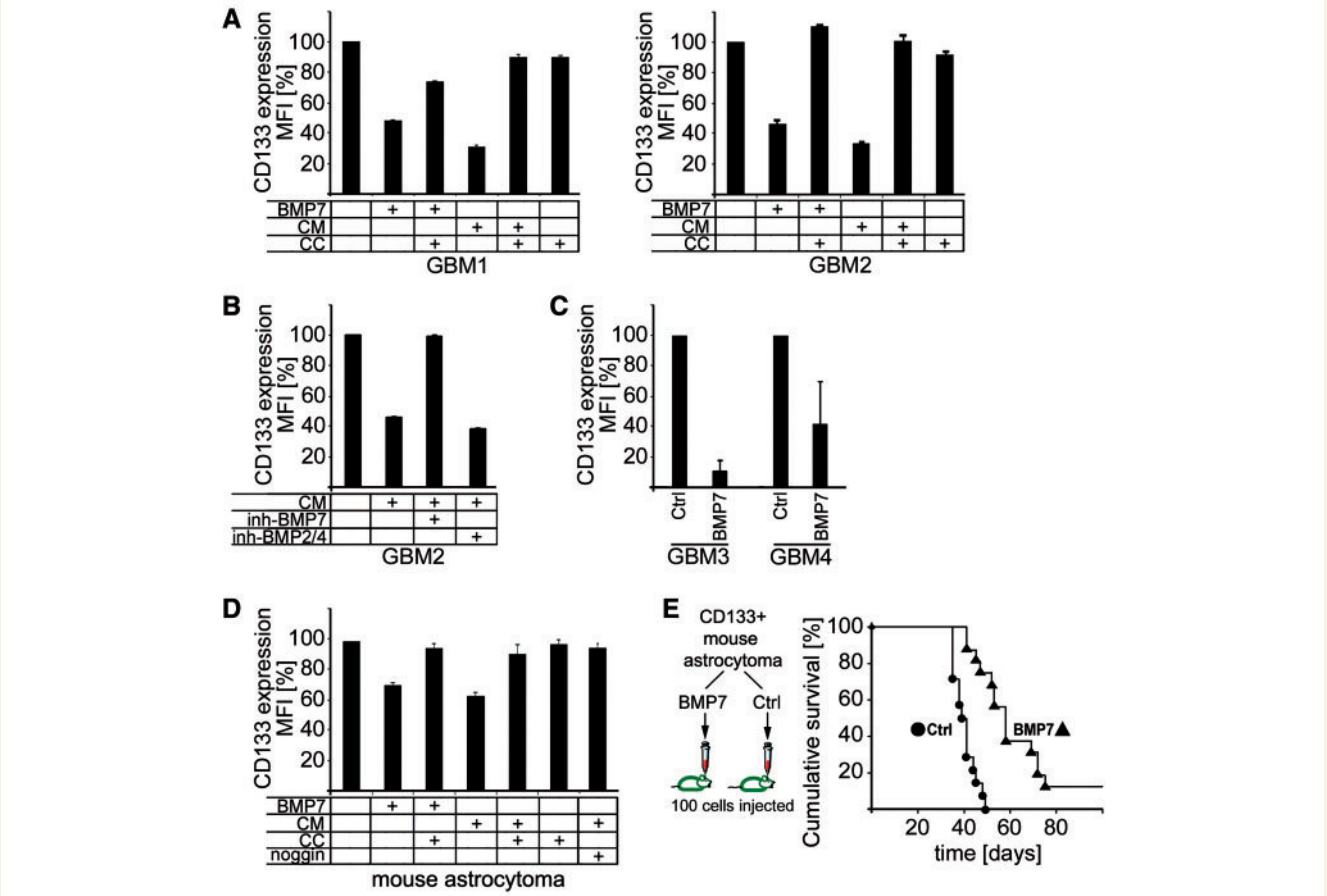

Figure 3 Neurosphere-derived BMP7 induced loss of CD133(+) GSCs from human glioblastoma and murine astrocytoma cultures. GSCs from GBM1, GBM2, GBM3 and GBM4 were stimulated with BMP7 or neural precursor cell-conditioned medium (CM) for 5 days, with or without the BMP-receptor antagonist compound C (CC); controls (ctrl) were maintained without stimulation or received compound C alone; CD133(−) immunolabelling [mean fluorescence intensity (MFI) for CD133] was evaluated by fluorescence-activated cell sorting (background-label was determined by isotype controls). (**A**) The GSC marker CD133 is attenuated after stimulation with BMP7 and neural precursor cell-derived BMPs in GBM1 and GBM2. (**B**) The neural precursor cell-conditioned medium-induced reduction in CD133 can be blocked specifically with an inhibitory antibody for BMP7 (7-inh-AB), but not with a blocking antibody for BMP2 and BMP4 (2/4-inh-AB). (**C**) CD133(+) GSCs from GBM3 and GBM4 are also reduced by BMP7. (**D**) Mouse astrocytoma cells were analysed for CD133(+) expression after addition of BMP7 or neural precursor cell-derived BMPs, as described above; note that noggin antagonized the neural precursor cell-conditioned medium-induced reduction of CD133(+) GSCs. (**E**) CD133(+) GSCs were purified from mouse astrocytoma cultures and either challenged with BMP7 or left untreated. Reduced capacity for tumour initiation was observed in BMP7 treated astrocytoma after orthotopic implantation of 100 cells into syngenic mice.

tumour-suppressor function in the CNS. We used mice expressing GFP under a modified promoter construct for nestin (nestin-GFP mice) to identify the endogenous neural precursor cells of the tumour host. We injected fluorescently-labelled, syngenic astrocytoma cells into the brain (caudate putamen) of young, postnatal Day 30, nestin-GFP animals and observed attraction of neural precursor cells from the subventricular zone to the tumour 14 days after the injection (at postnatal Day 44; Walzlein *et al*., 2008). By performing immunohistochemical colocalization studies and confocal microscopy we found that neural precursor cells in the margin abundantly express BMP7 (Fig. 5A and B); note that the BMP7-labelling was largely restricted to the rim of nestin-GFP positive cells (also designated as tumour-border) that surround an experimental brain tumour in many cellular layers, and was virtually absent from the main tumour mass and the tumour-free parenchyma. The BMP7 positive neural precursor cells largely co-labelled for polysialic acid-neural cell adhesion molecule (Supplementary Fig. 4A). Polysialic acid-neural cell adhesion molecule and BMP7 co-expressing neural precursor cells could also be detected in the anterior subventricular zone (Supplementary Fig. 4B). In addition, BMP2 and BMP4 were expressed in the stem cell niche, as already described by others (Colak *et al*., 2008), but were very rarely detected at the tumour border (Supplementary Fig. 4C).

We found that the extent of the neural precursor cell-response to brain tumours is age-dependent: in younger mice (postnatal Day 44) many neural precursor cells (95 cells per counting-frame) accumulated at the tumour, but in older mice (injected with astrocytoma at postnatal Day 180 and analysed 14 days after the operation e.g. postnatal Day 194) only an average number of

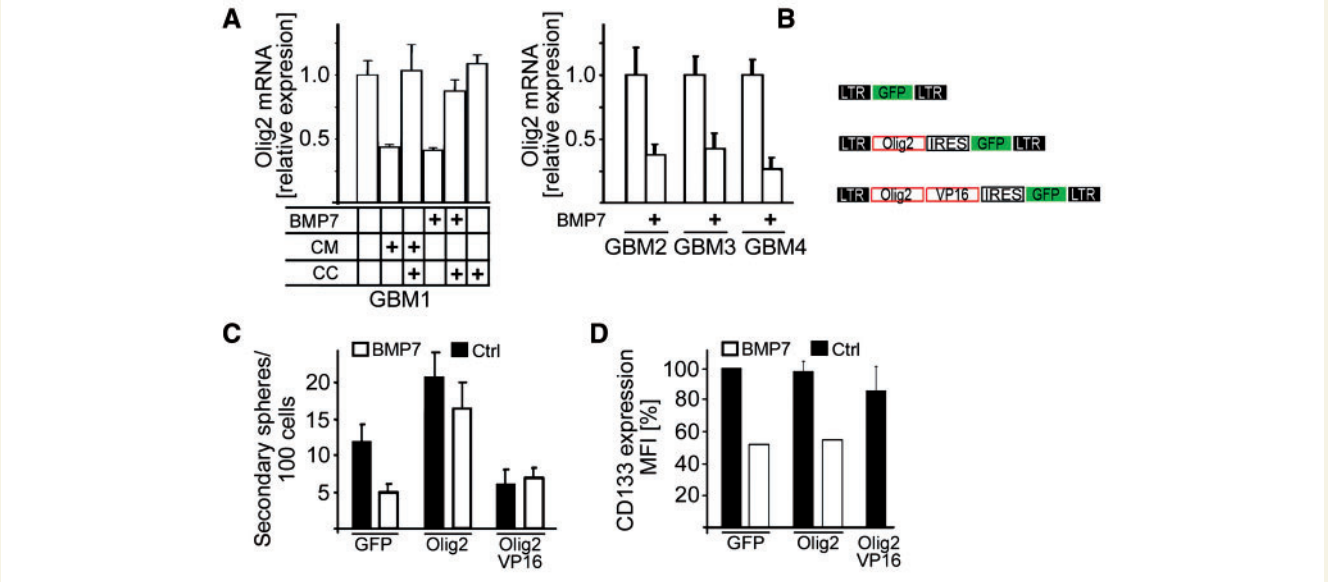

Figure 4 Neural precursor cell-derived BMP and BMP7 attenuate stem-like glioblastoma cells expansion via down-regulation of Olig2. (**A**) GBM1 GSCs were stimulated with recombinant BMP7 or neurosphere conditioned medium (CM) for 5 days, with or without the BMP-receptor type-Ia and Ib antagonist compound C (CC), respectively; controls (ctrl) were maintained without stimulation or received compound C alone; then expression of Olig2 was measured by real-time PCR; likewise the expression of Olig2 was measured in GBM2, GBM3 and GBM4 GSCs with and without stimulation by BMP7. (**B**) Olig2 expression and function in GSCs were directly manipulated with lentiviral vectors encoding GFP (control), Olig2 or dominant-negative Olig2 (Olig2-VP16); transduced cells were identified by bicistronic expression of GFP. (**C**) GBM1 cells expressing GFP, Olig2 or Olig2-VP16 were purified by fluorescence-activated cell sorting and maintained under control conditions or received BMP7 for 5 days, then the capacity for secondary sphere formation in normal medium was measured. (**D**) GBM1 cells were transduced as in **C**, subsequently CD133(−) surface labelling was measured in GFP expressing cells; note that Olig2 has virtually no influence on intensity of CD133(−) labelling.

15.5 cells per area were detected (Fig. 5C; see also Glass *et al*., 2005; Walzlein *et al*., 2008). However, the fraction of BMP7-expressing neural precursor cells remained high in both age-groups; we observed that ~58% of the tumour-associated nestin positive cells labelled for BMP7 in mice at postnatal Days 44 and 194. The immunohistochemical localization of BMP7 expression in tumour-associated neural precursor cells was confirmed in another animal model (Godin *et al*., 1998). We orthotopically implanted astrocytoma cells into syngenic mice, which express β-galactosidase under the promoter for BMP7. The BMP7-related reporter gene activity was detected in polysialic acid-neural cell adhesion molecule positive neural precursor cells in the tumour margin (Fig. 5D).

Taken together our data show that neural precursor cells in the brain tumour margin express BMP7, which we characterized as a paracrine tumour suppressor that specifically targets GSCs. The young brain recruits larger numbers of endogenous neural precursor cells to astrocytomas than the older brain, implying that only in the young brain neural precursor cells can efficiently suppress GSCs by activating Smad1/5/8 signalling.

Neural precursor cells induce Smad1/5/8 signalling in GSCs *in vivo* and reduce GSC-tumourigenicity

To investigate if tumour-associated neural precursor cells may have a tumour-suppressive action on GSCs *in vivo*, we asked whether neural precursor cells can evoke BMP-specific responses in human stem like glioblastoma cells. To address this question, GBM1 cells were inoculated into the brains of immunodeficient mice (Fig. 6). One group of glioblastoma-recipient mice was young (inoculated with GBM1 cells at P30 and inspected at postnatal Day 44; Fig. 6A–D). A second group of tumour-recipients was older (postnatal Day 194 at the end of the experiment; Fig. 6E). Four days before ending the experiment, some of the older mice were additionally injected with neural precursor cells into the tumour-area. At the end-point of the experiment both groups had developed glioblastomas, which were inspected for activated (phosphorylated) Smad1/5/8 (p-Smad1/5/8) in the GBM1 cells. GBM1 cells were identified by co-labelling with a human-specific antibody for vimentin [hu-vimentin; a radial glia marker that is expressed in human CD133(+) stem-like glioblastoma cells (Zecevic, 2004; Loja *et al*., 2009)]. As a control we also studied animals that received mock-injections.

Adult subventricular neurogenesis requires BMP signalling, which can be visualized by immunolabelling for p-Smad1/5/8 (Colak *et al*., 2008). Other brain regions, e.g. the caudate putamen in both young and old mice, were virtually free of p-Smad1/5/8 labelling (Fig. 6B and C). However, in young mice, glioblastoma in the caudate putamen showed intense staining for p-Smad1/5/8 (Fig. 6C and D) and colocalization with human-vimentin revealed that p-Smad1/5/8 staining largely originated from the tumour cells (Fig. 6D). Glioblastoma in older mice did not exhibit much p-Smad1/5/8 labelling (Fig. 6E), unless the

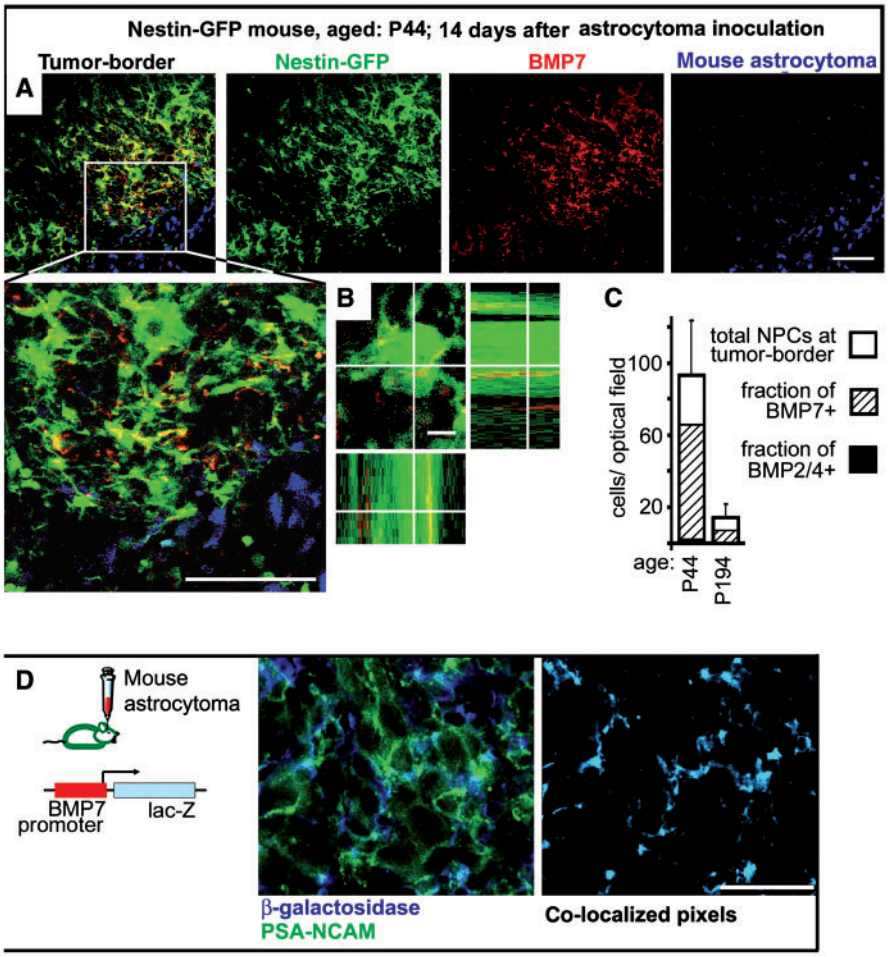

Figure 5 Endogenous neural precursor cells accumulate at experimental brain tumours and express BMP7. Nestin-GFP mice (aged postnatal Day 30 or 90) were inoculated with fluorescently labelled or unlabelled syngenic astrocytoma cells. Fourteen days after tumour-injection [mouse age is now postnatal Day (P) 44 and 194, respectively], brains were inspected for tumour-associated neural precursor cells and expression of BMPs. (**A**) GFP-expressing cells accumulate in large numbers at a brain tumour; BMP7 is abundantly expressed in and immediately around GFP-positive cells and in the tumour border; BMP7 labelling is absent from the normal parenchyma and from the tumour core; the boxed area is magnified. (**B**) Optical section obtained by confocal microscopy were processed to generate a 3D view of nestin-GFP(+) cells co-expressing BMP7 in the tumour border. (**C**) Quantification of the number of neural precursor cells (NPCs) accumulating at an astrocytoma in young and aged mice (bar diagram) and fraction of tumour-associated neural precursor cells expressing BMP7, respectively, BMP2 or BMP4. (**D**) Inoculation of syngenic astrocytoma cells into BMP7-reporter mice (BMP7 promoter driving the lac-Z gene), revealed that polysialic acid-neural cell adhesion molecule (PSA-NCAM) positive neural precursor cells express BMP7 in the tumour margin. Scale bars are: 100 μm in **A**; 5 μm in **B**; **C** statistical difference is significant, $P<0.001$.

older mice had been co-inoculated with neural precursor cells into the tumour-area. Quantification of p-Smad1/5/8 labelling suggested that stem-like glioblastoma cells respond to neural precursor cell-derived BMP *in vivo*: in postnatal Day 44 mice, many neural precursor cells accumulate in the tumour-border and induce BMP signalling in the GBM1 cells; in postnatal Day 180 mice, the few neural precursor cells in the tumour-vicinity induce very little Smad1/5/8 phosphorylation; and BMP signalling in glioblastoma of older mice could be restored after injection of neural precursor cells into the tumour (indicating that neural precursor cells induce Smad-1/5/8 activation in GBM1 cells). Mock injections were without effect (Fig. 6F).

Furthermore, we investigated if neurosphere-induced Smad1/5/8-activation in GSCs would have an effect on tumour initiation and survival in mice. We added neural precursor cell-conditioned medium with or without the BMP-receptor antagonist compound C to GBM1 stem-like glioblastoma cells, left the cells under these conditions for five days and then implanted 100 cells into brains of immunodeficient mice. We observed that GBM1 cells that had received neurosphere conditioned medium alone had a strongly reduced capacity for tumour-initiation, as compared to human stem-like glioblastoma cells incubated with neural precursor cell-conditioned medium plus compound C. Six out of 12 mice inoculated with GBM1 cells exposed to neural precursor

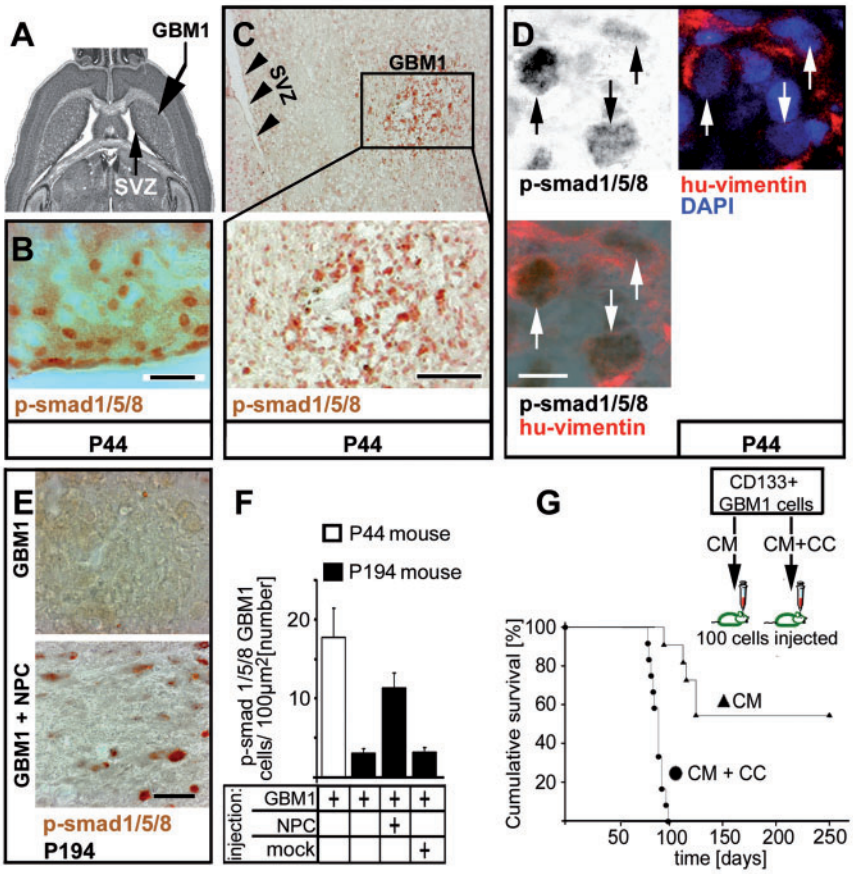

Figure 6 Neural precursor cells induce BMP-related Smad-signalling in GBM1 stem-like glioblastoma cells *in vivo.* Brains of young [postnatal Day (P) 44] and older (postnatal Day 194) mice were orthotopically implanted with GBM1 cells and inspected for Smad1/5/8 signalling after 14 days of tumour growth (**A–E**). The extended neural precursor cell (NPC)-induced Smad-signalling was quantified and the impact of neural precursor cell-derived BMP on glioblastoma-pathology was measured (**F** and **G**). (**A**) Outline of the area for tumour-inoculation and subventricular zone (SVZ). (**B**) Immunolabelling for p-Smad1/5/8 in the subventricular zone. (**C**) P-Smad1/5/8 labelling in the tumour-bearing hemisphere at postnatal Day 44; note that the tumour (boxed-area) but not the surrounding parenchyma label for p-Smad1/5/8; the boxed area was magnified. (**D**) The p-Smad1/5/8(+) cells are of human origin (GBM1 cells co-label with a human-specific antibody for vimentin; hu-vimentin). (**E**, upper) At postnatal Day 194 very little p-Smad1/5/8 labelling is detectable in GBM1-cell induced glioblastoma. (**E**, lower) Older mice were injected with GBM1 cells and (4 days before sacrifice) co-injected with neural precursor cells (into the glioblastoma), p-Smad1/5/8 labelling at end of experiment is shown. (**F**) Quantification of the number of p-Smad1/5/8-labelled GBM1 cells (identified by hu-vimentin) in young mice (postnatal Day 44), old mice (postnatal Day 194) and old mice substituted with neural precursor cells; mock injections (no injection of cells) served as controls for the substitution with neural precursor cells in older mice. (**G**) CD133(+) GSCs were purified from GBM1 and stimulated with neural precursor cell-conditioned medium (conditioned medium) with or without the BMPR-I antagonist compound C (CC). Reduced capacity for tumour initiation (after orthotopic implantation of 100 cells into non-obese diabetic-severe combined immunodeficiency mice) was observed only when GBM1 GSCs were treated with conditioned medium alone. Scale bars are: 20 µm in **B** and **E**; 125 µm in **C**; 50 µm in the magnified image; 5 µm in **D**; statistical difference in **F** and **G** is significant, $P<0.05$.

cell-conditioned medium survived free of symptoms until the end of the study (more than 240 days; Fig. 6G). In contrast, all mice receiving GBM1 cells treated with neurosphere conditioned medium plus compound C died within an average time of 48 days, All differences in survival time were statistically highly significant ($P<0.001$).

Overall, our data show that neural precursor cells release BMP in the tumour margin and can suppress glioblastoma by targeting GSCs. The data presented here now explain previous observations by us and others that co-inoculation of neural precursor cells with human or mouse glioblastoma cells reduces tumourigenicity and improves survival (Staflin *et al*., 2004; Glass *et al*., 2005; Suzuki *et al*., 2005; Walzlein *et al*., 2008).

Discussion

The germinal centres of the CNS are thought to be the point of origin for primary brain tumours (Holland, 2001; Sanai *et al*., 2005; Stiles and Rowitch, 2008). Neural precursor cells are the mitotically and migratory active cells of the brain and genetic mutations can transform them into a highly proliferative and invasive

cellular mass (Holland, 2001). The sub-fraction of glioblastoma cells with stem cell-like properties may arise either early during tumourgenesis or may accumulate while the tumour progresses into more malignant stages (Jordan, 2009); in any case GSCs appear to control the course of pathology (Gunther *et al*., 2008). In extension to this concept, we hypothesize that neural precursor cells are not only a source of glioblastoma but also serve as line of defence against glioblastoma—since they can suppress GSCs.

In our present study we have shown that neural precursor cells accumulating at brain tumours in the young brain abundantly express BMP7, but not BMP2 or BMP4. It has been demonstrated that recombinant BMP2 and BMP4 may function as GSC-specific glioblastoma therapeutics (Piccirillo *et al*., 2006; Lee *et al*., 2008). Our study suggests the possibility that BMP7 is an endogenous, GSC-specific anti-tumour factor, which can be therapeutically useful by the same criteria used for BMP2 and BMP4. Neural precursor cell-released BMP7 stimulated a canonical BMP response in stem-like glioblastoma cells, which interfered with all three key-functions of GSCs (Jordan *et al*., 2006; Jordan, 2009): (i) the ability of GSCs to maintain a cellular hierarchy was reduced (the markers or undifferentiated cells CD133, nestin and Olig2 were lost, whereas the differentiation marker GFAP was induced); (ii) the ability for self-renewal was reduced (attenuated ability to form spheres); and (iii) the potential for tumour-initiation by murine and human CD133-positive GSCs was strongly reduced.

The effect of neural precursor cell-conditioned medium was due to BMP7 since the reduction in GSC number and the induction of GSC differentiation were blocked by a BMP7 inhibitory antibody; as well as by compound C, a blocker of canonical BMP signalling (Yu *et al*., 2008) and noggin, a natural BMP-antagonist (Massague, 2000). We found that BMP-release from neural precursor cells down-regulates Olig2 in GSCs—a transcription factor that is required for tumour initiation in the CNS (Ligon *et al*., 2007)—and thereby reduces tumour expansion. Our data imply that the BMP-Olig2 signalling axis may have different functions during physiology or pathology, which are lineage determination (Lim *et al*., 2000; Colak *et al*., 2008), respectively, tumour suppression.

The subventricular zone is the likely source for primary glioblastoma (Marumoto *et al*., 2009). We speculate that the subventricular zone of younger individuals is intrinsically protected against transformed stem cells by BMP and especially BMP7-release from neural precursor cells. However, this intrinsic tumour-suppressor mechanism is lost with the age-related decline in neurogenesis. One of the few known risk factors for glioblastoma-development is ageing. We hypothesize that the older brain may have a higher propensity to develop primary glioblastomas when the number of BMP-secreting neural precursor cells is reduced. Additionally, BMP7 is a target for epigenetic inactivation in high grade primary brain tumours (Ordway *et al*., 2006). In our future studies we will address if BMP7 loss is an early event during gliomagenesis, which may point to a pro-pathological role for BMP7-silencing during neoplastic transformation of stem and precursor cells in the brain.

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Supplementary material

Supplementary material is available at *Brain* online.

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